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The Normal and Pathological Histology of the Ventriculus of the Honey Bee, with Special Reference to Infection with Nosema apis

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THE NORMAL AND PATHOLOGICAL HISTOLOGY OF THE VENTRICULUS OF THE HONEY-BEE, WITH SPECIAL REFERENCE TO INFECTION WITH *NOSEMA APIS*

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In the great amount of work which has been done on the organisms of insect-borne diseases and on micro-organisms found as parasites in insect tissues, the pathological histology of the insect itself has been singularly neglected. Furthermore it is found that the basis for such study—namely, a knowledge of the normal histology and cytology—is often almost entirely lacking. The fact that many of the intracellular symbionts of insects and the granule-like Rickettsia organisms of typhus and trench fever, were for some time after their discovery confused with normal cell inclusions, well illustrates the need for study of the normal histology and particularly of the granular cell inclusions universally present in insect tissues. While the primary purpose of this paper is a consideration of the pathology of the adult honey-bee, there has necessarily been included as a basis therefor, a somewhat extended discussion of the normal histology and cytology. This broader phase of the problem it is believed will appeal to the parasitologist or other worker with micro-organisms, quite as strongly as the pathology itself.

THE DISEASES OF ADULT HONEY-BEES

The diseases of bees best known to the beekeeper, and those ordinarily referred to under the term "bee diseases," especially in America, are the diseases of the larvae or brood. Of these the most common and the most serious are the bacterial diseases, American foulbrood and European foulbrood. In addition to the larval diseases, there are a great number of disorders of the adult honey-bee, accounts of which are found in beekeeping literature as far back as three hundred years. (Bullamore 1922; Zander 1911, 1921.) These disorders, nearly all of obscure etiology, are characterized by a variety of symptoms and are known to the beekeeper under a wide variety of names. Besides those conditions supposedly due to old age, exposure, insufficient or improper food, or poisoning, may be mentioned dysentery, diarrhoea, Ruhr, May-sickness, May-pest, June-sickness, paralysis, palsy, trembling, dizziness,

vertigo, spring dwindling, disappearing disease, dropsy, Sandläuferei, Fussgängerei, Isle of Wight disease, Nosema-disease, Aspergillusmycosis and "paratyphoid." The variety of symptoms exhibited in these disorders is indicated to some extent by their names. A review of the literature reveals the very greatest confusion as to nomenclature, the symptoms associated with a given disease, and etiology. Despite the multiplicity of names and the variety of symptoms, these disorders are all marked by one outstanding feature—namely: the affected bees, unable to fly, are found crawling or nearly motionless, usually near the hive, where they die after a few hours.

While these disorders may occur at any time of the year, they are most common in late winter and early spring, being known frequently as winter losses, spring dwindling, May-sickness, etc. They appear to be highly contagious in some cases and in others not at all so. There is no constant correlation of any of the disorders with such external factors as periods of unfavorable weather or the blossoming of certain nectar- or pollen-producing plants.

It is probable that many of the disorders which have been considered distinct pathological entities are in reality only symptoms of diseases which may be evidenced externally by a number of different conditions. This is well illustrated in the literature of Isle of Wight disease and of Nosema-disease, the best known of the diseases of adult bees, in which the external symptoms of nearly all the various disorders have been described at one time or another as characteristic of each of these two diseases. Indeed it would appear that the various symptoms are not specific for any disease but are evident whenever the bees are weakened from any cause. The causes of such weakening are not known except in the few cases where definite organisms have been described. The stricken bees may exhibit marked abdominal distention with copious defecation on combs, hive or ground, this condition being known as dysentery or Ruhr. In "dry dysentery" or "constipation," there may be abdominal distention with apparently no defecation. The feces may be thin and watery, or thick and ropy, light or dark in color and acrid or otherwise in odor. The bees appear weakened, and are found mostly on the alighting board or on the ground in front of the hive, singly or in groups, clinging to spears of grass, lying nearly motionless on the ground, or crawling about either actively or sluggishly. The bees are unable to fly, though they may leave the ground for a flight of a few inches, their progress being then a combination of active crawling and "hopping." At times certain of the legs seem to be paralyzed and are dragged along in crawling. The wings are often "dislocated," i. e., the hind wings are not hooked to the fore wings, and are capable of only an irregular trembling motion, or at best a feeble, jerky fanning.

In crawling, the bee may hold itself as if bent to one side, and may describe small circles. The terms paralysis, trembling, palsy, vertigo, etc., are used to designate these various conditions.

Nosema-disease (Nosemaseuche) is the term proposed by Zander (1911) to designate an infectious form of Ruhr (dysentery) and Maikrankheit (May-sickness), with both of which he found associated the Microsporidian, *Nosema apis,* described by him in 1909. Zander considered copious defecation the surest sign of Nosema-disease, though this symptom was frequently absent. The sudden appearance of groups of bees dying inside or outside the hive was held to be an important characteristic. White (1919) studied Nosema-disease in America and concluded that it is an infectious, though not particularly malignant disorder of the adult bees. The parasite is cosmopolitan in its distribution. In heavy infections the colonies become weak and may be destroyed. The behavior of the infected colony is similar to that of a healthy one. The individual infected bees manifest no external symptoms until actually dying, when they become unable to fly and crawl about as described above. Diagnosis of the disease is confirmed by finding the spores of the parasite in the ventriculus.

Isle of Wight disease, named from the epidemic which was first reported from the Isle of Wight in 1906, has caused great losses in the British Isles. The disease was first described as "paralysis" and "dysentery" from the symptoms most commonly observed. However, it was soon shown that these symptoms were not invariably present. Indeed the only constant symptoms were the inability of the stricken bees to fly and their ultimate death. Fantham and Porter (1912a, 1912b) in their studies on Isle of Wight disease found *Nosema apis* in more or less constant association with the disease, and together with other British workers, considered this organism to be the cause of Isle of Wight disease (Graham-Smith, Fantham et al., 1912). Rennie and Harvey (1919a, 1919b), however, while holding Isle of Wight disease to be infectious, concluded that *Nosema apis* was not causally related thereto. This has apparently been confirmed by the work of Rennie, White and Harvey (1921) who found the thoracic tracheae of diseased bees containing numbers of the mite *Tarsonemus woodi,* which they considered the cause of Isle of Wight disease. These workers have also discussed the relation of the mite to the pathology of the disease.

A number of other presumably infectious disorders of adult bees associated with micro-organisms, or otherwise, may be mentioned. In Brazilian bee-pest the bees die in great numbers as in Isle of Wight disease and Nosema-disease. While the disease has been attributed to poisonous nectar, the cause remains unknown (Zander 1911). Maassen (1916a) mentioned Aspergillusmycosis, a disease affecting both larvae and adults, caused by a species of Aspergillus. Zander (1911, 1921)

and Maassen (1919) both mentioned the larvae of a Meloid beetle, *Meloë variegatus,* as at times causing losses of adult bees. The meloid larvae are found attached to the intersegmental membranes of the abdomen, having been picked up by the bees in foraging. Maassen (1919) also reported the finding of an ameba-like organism in the Malpighian tubes of adult bees, which in one case at least was associated with the death of many bees. Bahr (1919) found a bacillus of the paratyphoid group in the intestine of adult bees which were unable to fly and which were dying in numbers. Feeding pure cultures of this organism reproduced the disease. Nosema was not found in connection with this disorder.

In America and Australia serious losses of adult bees have occurred for which no satisfactory cause could be assigned, the symptoms being similar to those described for Isle of Wight disease and Nosema-disease. *Nosema apis* is known to occur in these countries and may be partly responsible for this damage, though the presence of Nosema has not been demonstrated in all cases.

It is thus seen that there are a number of disorders in which the adult bees die in numbers, exhibiting a variety of symptoms. In some cases organisms are known to be associated with, and are perhaps the cause of, the disease. In others the cause can not be designated with certainty. None of these disorders is characterized by any outward symptom definitely diagnostic for that disease. In all cases the stricken bees are unable to fly and die in numbers. It is possible, as suggested by several investigators, that inability of the bees to fly and various other conditions accompanying their death and cited as symptoms of different diseases, may represent merely the final stage in the weakening of the bees due to whatever cause.

It would accordingly be possible to have any number of disorders of adult bees, or rather disorders due to any number of different causes, in which the outward symptoms would be the same. The evidence already cited seems to indicate that such is the case. Since external symptoms are nearly valueless in indicating the real nature and causes of diseases of adult bees, the study of internal pathological conditions would seem to promise the best basis for accurate diagnosis. This method has been pursued in certain bee diseases with which an organism is definitely associated, as in Nosema-disease. The emphasis, however, has been placed on determining the presence or absence of the parasite rather than on the condition produced in the host tissues. Even here the possibility of factors other than the parasite contributing to the pathological condition is not precluded. The fact that many stricken bees contain but a few parasites, while other apparently healthy bees are heavily infected, indicates that other factors do operate in Nosema-disease. If analysis of factors other than the known or suspected

organism is necessary in disorders where such an organism has actually been demonstrated, the need for such study is all the more imperative in those disorders of adult bees of which the cause is wholly unknown or in those which have been attributed to such indefinite factors as long winter confinement, bad weather, and poisonous pollen or nectar. A comparative study of the histology and cytology of both healthy and diseased bees would seem to be one of the most promising methods of attacking the problem.

No group of animals furnishes examples of parasitism greater in point of number and diversity of nature than the insects and other arthropods. Nevertheless most of our knowledge of these parasites concerns only the organism itself, singularly little being known of the effect of the parasite on the host. In many cases in which a parasite causes a disease of economic importance the disease itself is well understood as concerns symptoms, the causal organism, the manner of transmission, and the methods of control, while little attention has been given to the pathological histology of the host. Beyond certain of the grosser and more obvious features, the changes brought about by the parasite in the host cells and tissues and the disturbances of their functions are almost unknown.

This lack of knowledge of pathological histology is especially marked in connection with diseases of the adult honey-bee, in which the diseased conditions themselves are not well understood. As a preliminary investigation of the pathology of the honey-bee from the histological point of view the present study was undertaken with the purpose of determining what changes are brought about in the tissues and cells of the ventriculus by the presence of *Nosema apis*, and further of determining what pathological conditions this organ may exhibit in certain disorders not associated with the presence of *Nosema apis*.

MATERIALS AND METHODS

Material was obtained chiefly from the University apiary, University Farm, St. Paul, during the years 1916-17 and 1919-21, and from Cornell University apiary at Ithaca, 1917-18. Bees were taken mostly from the hive entrance, though occasionally from within the hive or from blossoms. The bees were dissected as soon after their capture as possible, usually within an hour or two. After the head was cut off, the entire digestive tract was withdrawn by seizing the tip of the abdomen with forceps and pulling gently. The ventriculus was separated from the hind-intestine and dropped into 0.75 per cent. sodium chloride solution or into Locke's solution. Another method of obtaining the ventriculus was to cut off the entire abdomen and drop it into physiological saline solution. With fine forceps sufficient of the

chitinous covering was torn away to enable the ventriculus to be removed easily. Live bees were used in all cases in order to avoid any possible effects of chloroform or other killing agent.

In examining the ventriculus in the fresh condition, the whole or a small portion of the organ was crushed beneath a cover-glass. The best results were obtained by using small portions prepared as follows: A small section, about one-half a millimeter in thickness, was removed by means of scissors, this section appearing as a small white ring (the ventriculus wall) with a brownish gelatinous mass (the peritrophic membranes) within. This brownish mass was removed entire with forceps, leaving the wall intact; or the wall itself was cut with scissors, whereupon it would turn inside out, separating itself almost entirely from the peritrophic membranes. In this manner a portion of the wall of the ventriculus could be mounted free from its contents. Hanging drops were also made by touching the cut end of the ventriculus to a cover-glass and inverting this over a hollow-ground slide. The latter method allowed prolonged examination without changes in location or appearance of the material due to drying.

Cover-glass preparations were made usually as shallow hanging drops which were observed in the fresh condition and then fixed by being dropped flat upon the surface of sublimate-alcohol, or placed over a Van Tieghem cell containing 2 per cent. osmic acid solution, and then hardened by dropping upon 70 per cent. alcohol. By the latter method the preparation could be watched during fixation. Thus by noting its location a given cell could be studied in the fresh condition, during fixation and after staining.

Sections 3 to 5μ thick were made from material fixed in sublimate-alcohol, Bouin's fluid, Zenker's fluid, Gilson's mercuro-nitric solution, Hollande's (1912) bichromate-formol solution and in osmic acid vapor. The stains used for sections were mostly Heidenhain's iron-hematoxylin, counterstained with eosin, Delafield's hematoxylin, and Romanowsky stain.* For cover-glass preparations the most successful stains were Giemsa (Gruebler's Giemsalösung) and Romanowsky.* The preparations were left in the stain from 15 minutes to 24 hours, washed in distilled water, dehydrated rapidly in 95 per cent. alcohol or in acetone, passed through xylol and mounted in balsam or cedar oil.

THE NORMAL VENTRICULUS

Determination of the changes produced in the ventriculus by the presence of parasites, or the histological diagnosis of any other pathological condition, must be preceded by consideration of the normal

*"Romanowsky" stain, obtained from Noyes Brothers & Cutler, St. Paul, prior to 1915: equal portions of two stock solutions, "eosin" solution and "methylene blue" solution, diluted 1 to 10 in distilled water.

ventriculus, or rather the range of different conditions exhibited by this organ taken from apparently healthy bees. The criterion by which a bee is to be judged "healthy" or otherwise, is necessarily indefinite, since bees heavily infected with parasites may be outwardly indistinguishable from uninfected bees, while other bees may die in numbers from no apparent cause. Until more exact information is available concerning disorders of adult bees, "healthy" bees may be considered those in which neither external nor internal pathological conditions are apparent and which are obtained from a strong, vigorous colony, or at least one not suffering any marked loss of bees.

The ventriculus, or mid-intestine, lies in the anterior and dorsal portion of the abdomen. It is a more or less U-shaped tube, of uniform diameter, with numerous annular constrictions. It is ordinarily yellowish or whitish translucent in appearance, with reddish brown contents which give the whole a reddish brown color, though this varies somewhat from pale yellowish brown to dark brown. A deep constriction, the proventriculus, separates the ventriculus at its anterior end from the crop or honey-sac. About the ventriculus is the tangled mass of whitish or yellowish Malpighian tubes which empty into the digestive tract at the junction of the ventriculus and the small intestine, the latter being a coiled tube of about one-third the diameter of the ventriculus.

The structure of the ventriculus has been described by a number of workers (Frenzel 1886, Snodgrass 1910, White 1919, Pavlovsky and Zarin 1922). The wall of the ventriculus is made up of a number of structures. Forming a network on the outer or coelomic surface are three layers of muscle fibers, the outer and inner layers being longitudinal and the middle layer transverse. Within the muscle layers is the basement membrane which is continuous anteriorly with that of the fore-intestine (esophageal valve) and posteriorly with that of the Malpighian tubes and small intestine. Attached to the basement membrane are the cells composing the epithelium. The epithelial cells possess many fine, more or less parallel hairs arising from their surface and extending into the lumen of the ventriculus, these constituting the striated border. In the lumen and extending the entire length of the ventriculus are the peritrophic membranes, concentrically arranged. The outer, and hence most recently formed, peritrophic membrane either lies free in the lumen, sharply differentiated from the epithelium, or merges gradually with the substance of the striated border.

The inner surface of the epithelium is not regular, but appears in section to be studded with projections or villi, between which are pits or crypts (Fig. 1). The projections are joined to each other forming a honey-comb of walls surrounding the cylindrical crypts. At the bottom of the crypts are the nidi composed of the many small regen-

eration cells, while the walls are composed of columnar or elongate-pyriform cells. Each of the latter is attached to the basement membrane by a narrow stalk, while the free end tends to be as nearly spherical as the surrounding cells permit. The total thickness of the epithelium averages approximately 70μ, with extremes of 30 and 110μ. At the bottom of the crypts the epithelium measures about 25μ in thickness.

The striated border forms a layer lining the inner surface of the epithelium and more or less completely filling the crypts. That this layer is made up for the most part of many fine hairs, is seen in sections of material fixed in various solutions. In addition the hairs may be demonstrated in almost every fresh mount and are striking in appearance. They radiate from one side or at times apparently from the entire periphery of the isolated cells (Figs. 10, 18-21).

Lying over the striated border and uniting the free ends of the hairs extends a membrane of varying thickness and somewhat indefinite outline, staining like the substance of the striated border, though more intensely, as if it were a condensation of that substance (Figs. 1, 3, 6 and 8). This membrane is apparently the peritrophic membrane in process of formation, since it may frequently be demonstrated to be continuous with a well defined peritrophic membrane which has become detached from the epithelium in another portion of the ventriculus. While a number of workers have considered the peritrophic membranes to be the product of certain cells at the anterior end of the mid-intestine (see discussion in Deegener, 1913), it would appear from the writer's observations which in general agree with those of Pavlovsky and Zarin (1922) that the peritrophic membranes of the honey-bee are formed from the substance of the striated border, together with the remains of cells which are given off from the epithelium in the process of digestion, and that this formation takes place not directly at the surface of the cells but at the free ends of the hairs forming the striated border. Snodgrass (1910) does not mention the hairs, but figures an "intima" with a "gelatinous mass" between the intima and the epithelium, the gelatinous mass apparently representing the striated border. The "intima" or "cuticle" (Pavlovsky and Zarin) together with the striated border separate from the epithelial cells, shrivel and shrink and finally become peritrophic membranes. The process is repeated in the formation of each successive membrane. These lie one within another, appearing in section as thin, much wrinkled bands, somewhat refractive, sharply defined in some regions, in others merging with a finely granular substance containing here and there remains of epithelial cells. The peritrophic membranes stain slightly or not at all, being usually yellowish in color, while the granular masses associated with them stain with eosin. In the fresh condition the peritrophic membranes are seen as a reddish brown, gelatinous mass, which on

traction with forceps separates easily from the epithelium. The innermost membrane forms a tube for the passage of pollen grains and other food, and at times portions of it pass down with the food and are found in the hind-intestine surrounding little pellets of pollen.

A long tubular fold of the fore-intestine projects into the lumen of the ventriculus, forming the esophageal valve. The basement membrane bears a layer of epithelial cells somewhat smaller than those of the ventriculus. Many of these cells are stalked like those of the ventriculus, and appear to be given off from the epithelial layer from time to time. While there is no sharp dividing line between esophageal valve and ventriculus, the cytoplasm of the ventriculus cells is markedly more deeply stained than that of the valve cells.

The contents of the ventriculus during the field season usually consist only of the peritrophic membranes and the gelatinous substance associated with them. A few pollen grains are found at times, though these apparently pass rapidly to the hind-intestine where they may be found in great numbers in all stages of digestion. When the bees are in winter quarters, or at times when cleansing flights are infrequent, the ventriculus may contain a considerable accumulation of pollen, particularly at the posterior end, together with feathered bee hairs and many micro-organisms, chiefly bacteria and yeasts. The solid portions of the contents are usually enclosed by the inner peritrophic membrane, there being no direct contact with the epithelium. Occasionally, however, there may be found in sections small groups of bacteria lying next the epithelium. Whether or not bacteria can make their way through several uninjured peritrophic membranes is not known.

The intestinal flora of the honey-bee includes a number of different bacteria and yeasts, some of which are almost constantly present (White 1906). The greater number of these organisms are found in the hind-intestine, particularly the rectum, relatively few both as to numbers and variety being found at any time in the ventriculus, except at those times when an accumulation of food material occurs as noted above. The great difference in bacterial content between mid- and hind-intestine is noticeable at once in fresh preparations, and is confirmed by broth and agar cultures. The writer has at times obtained no growth in media inoculated with a small section of wall and contents of the ventriculus, while very frequently no growth resulted in tubes inoculated with a small section of the ventriculus wall freed from the peritrophic membranes. The explanation for the slight bacterial content of the ventriculus lies perhaps in the fact that solid particles pass rapidly to the hind-intestine, and further that the contents of the ventriculus are at times rather acid, which may inhibit multiplication of organisms.

One of the most striking features encountered in examining the epithelium of the ventriculus is the extreme variation of the cells and

their nuclei as to size, shape and arrangement, and as to the character, size and number of the cytoplasmic inclusions. These variations are due not only to differences in location in the epithelium and to differences in age, as are found for example in the regeneration cells and the cells of the crypt walls, but probably also to differences in function in the process of digestion, not as yet well understood. It is correspondingly difficult, therefore, to determine just what cells are to be considered "normal" and those which may exhibit some pathological condition.

In the bee, as in most insects, cells or portions of cells are given off into the lumen of the ventriculus, these being known as secretion cells (Frenzel 1886) or sphaerocytes (Deegener 1913). These apparently contain digestive enzymes, which are liberated in the lumen of the ventriculus by the disintegration of the cells. Pavlovsky and Zarin (1922) have discussed the ferments of the digestive tract of the bee. The proliferation of the secretion cells takes place chiefly from the walls of the crypts and rarely from the nidi at the bottom. Snodgrass (1910) stated that these "discharged cellules" all contain nuclei, and believed their proliferation to be due to an active division of cells forming the walls and lips of the crypts. According to the writer's observations the secretion cells may arise in several ways:

1. In some cases it appears that the entire cell, the basal portion of which usually consists of a slender stalk, elongates greatly and finally separates completely from the basement membrane. Such a secretion cell at first differs from cells of the epithelium only in that it has become nearly spherical. This condition is seen occasionally in sections in regions where the epithelium is greatly thickened as a result of the elongation of the cells. That the individual cells are easily detached from the basement membrane is shown by the great number of large, spherical, nucleate cells, apparently uninjured, to be found in every fresh preparation (Fig. 10).

2. In both sections and fresh mounts, especially the latter, are always to be observed many spherical secretion cells without nuclei, which average somewhat smaller than the nucleate cells just described (Figs. 11-20). These may arise in several ways: A division of the cell body without nuclear division may take place, though this has not been observed. In fresh mounts there have been noted frequently, however, two or more cells, equal or unequal in size, joined together as shown in Figures 17 and 18. What is apparently the same condition has been observed in sections of the normal ventriculus undergoing great proliferation of secretion cells. Many elongate secretion cells bear on their distal extremities another secretion cell. Not uncommonly there are several cross-walls in the latter, indicating perhaps as many different stages in their formation. Whether a

division of the parent cell takes place or whether one cell is formed on the surface of the other by a process resulting in what may be compared to a blister, cannot be stated. The latter method seems probable from the fact that very frequently the "blister" is clear and homogeneous while the parent cell may contain a nucleus together with the characteristic granules described below. Both may contain such granules, however. That actual division of the parent cell does not take place is further shown in sections of the ventriculus infected with Nosema, in which the same or a similar process may at times be noted. Figure 7 shows several of the greatly elongate epithelial cells bearing on their free ends tiny cap-like cells, several of the latter containing cross-walls similar to those observed in uninfected specimens. From the fact that these tiny secretion cells contain no spores and that their cytoplasm stains much more intensely than that of the infected parent cells, it is evident that no division of the parent cell body has taken place, but rather that the cap-like cells have obtained the material of which they are composed through osmosis.

3. In nearly all fresh preparations occur isolated cells beraing a stalk. These vary in size from tiny cells a few micra in diameter to those as large as the average epithelial cell. They are without nuclei, their cytoplasm may contain the refractive granules characteristic of the epithelial cells, and the hairs of the striated border are usually, though not always, present (Figs. 19, 20). Occasionally a tiny cell of this sort is seen attached by its stalk to a larger cell which may also be stalked. As many as three or four tiny cells may be joined by their stalks, forming a chain. These recall the sphaerocytes described and figured by Deegener (1913) from the mid-intestine of the larva of *Deilephila euphorbiae*. In the honey-bee they seem to arise through a constricting off of the surface portion of the epithelial cells. The number of these stalked secretion cells varies greatly in different specimens; in some they are rare or absent while in others certain parts of the ventriculus are lined with them. The rôle which these various types of secretion cells play in the digestive processes, and the conditions which give rise to one type or another, are not understood.

The nuclei vary greatly in appearance, both in the fresh and stained condition. They are spherical, oval or rarely elongate. The nuclei of the nidi as seen singly or in groups in fresh preparations are spherical and resemble fused masses of small, more or less spherical, non-refractive granules. Frequently there are also to be observed in the nucleus one to several small masses, very slightly refractive, either spheroid or irregular in shape. The nuclei of the nidi stain more intensely on the whole than do those of the surrounding cells, and appear either as a mass of small spheroid granules, intensely stained,

or as a lightly stained granular area containing one to several large, deeply stained granules or masses with definite outlines. These latter apparently correspond to the slightly refractive masses seen in the fresh condition. Though more or less active division of these cells undoubtedly takes place, nuclei in division are rarely seen, either in fresh or stained material. Two nuclei are to be observed in cells occasionally, however. Figure 2 represents a regeneration cell nucleus with division, apparently by amitosis, nearly complete.

The nuclei of the cells forming the crypt walls when freshly dissected usually appear as spheres of a clear liquid in which are suspended a number of slightly refractive masses, spheroid or somewhat elongate. These masses may be few and comparatively large or small and very numerous. The clear body of the nucleus, which at times is not homogeneous but is indistinctly granular, may or may not be liquid. However, the suspended masses appear to be firmly held, since they never exhibit Brownian movement as do the granules in the cytoplasm. The nucleus seems to be less easily destroyed by mechanical means than the rest of the cell, since many isolated nuclei, quite intact, are to be found in fresh mounts. The appearance of the nucleus fixed in osmic acid vapor is generally little changed from that of the fresh nucleus, the body of the nucleus being homogeneous or finely granular, and staining lightly, while the suspended masses stain deeply (Fig. 3). Nuclei fixed by methods other than osmic vapor in general appear either coarsely granular or coarsely reticulate (Fig. 1). Lewis and Lewis (1915) obtained similar results in fixing chick embryo cells, finding that only with osmic acid vapor was the appearance of the fresh nucleus faithfully preserved, while other fixers yielded the reticulate "text-book" nucleus.

Another condition of the nucleus is often to be observed in any one of the different types of epithelial cells, in both fresh and stained preparations. The nucleus is spherical or oval and contains what appears to be a clear homogeneous liquid. Suspended in this liquid or lying at one side of the nucleus, is a dense, granular, spheroid or irregular mass, the granules being slightly refractive with indistinct outlines (Figs. 10, 22, 23, 25). When stained such nuclei appear as vacuoles containing a densely stained, granular body.

THE CYTOPLASM AND ITS INCLUSIONS

The cytoplasm itself, whether of the nucleate epithelial cells or of the discharged secretion cells, is generally a clear, colorless liquid, though at times it is indistinctly finely granular. Its liquid or semi-liquid condition together with the very thin, elastic cell membrane, allows the cell to assume almost any shape in response to pressure of its neighbors, the cell becoming spherical when the pressure is removed.

In the cytoplasm are suspended the refractive granules or other inclusions, these being frequently in active Brownian movement. In sections the cytoplasm appears finely granular. It may be evenly distributed throughout the cell, or may be gathered into fine, irregular strands forming a network. In cells fixed in osmic acid vapor occur tiny vacuoles representing the spherical granules which are destroyed in the process of preparing paraffin sections. With other fixers it is but rarely that any trace of the spherical granules, which are so conspicuous in the fresh condition, is found in the sections.

The cytoplasmic inclusions are among the most puzzling features of the epithelium. Chief among these are the highly refractive, spherical granules which are found in nearly every cell. Though granules resembling those of the honey-bee occur in the mid-intestinal epithelium of nearly all insects, the information to be found in the literature concerning such granules, is rather meager. In most descriptions of the intestine of various insects, these granules, where mentioned at all, are referred to as secretion granules or fat droplets, with no further discussion. Frenzel (1886) in his work on the mid-intestinal epithelium of a number of insects, including the honey-bee, has described and figured "Sekretkügeln" or "Safttröpfchen" found in the cytoplasm. They have also been noted by Petersen (1912). Koehler (1920) has considered in some detail the nature of certain of these bodies and their possible relation to digestion.

The cytoplasmic inclusions may be divided into several groups: (1) spherical, refractive granules, 1 to 3μ in diameter; (2) very tiny, refractive granules, spherical or somewhat irregular, 0.2 to 0.5μ in diameter; (3) irregular, slightly refractive bodies; (4) faint, non-refractive rods; (5) vacuoles. These groups are considered below:

1. Koehler (1920) in her work on the inclusions of the epithelial cells of the honey-bee's intestine and the related problems of digestion, discussed but one type of inclusion, the spherical, refractive granules measuring 1 to 3μ. These conspicuous bodies are found in greater or less numbers in almost every cell. They have been variously supposed to be secretion granules playing some rôle in digestion (Zander 1911), or reserve food material (Frenzel 1886). Koehler, as a result of various staining reactions and other microchemical tests, concluded that the granules are composed of some calcium compound, probably calcium carbonate, with an outer covering of some organic material. She believed they might indicate an excretion of calcium by the epithelium, or that they might play an important rôle in the neutralization of acids formed in digestion. These bodies may be found in any cell of the epithelium though the regeneration cells contain few or none (Figs. 10-12, 14, 16-23). The number to be found in any cell varies greatly. In some specimens almost every cell in the epithelium is

literally packed with granules, while in others there may be many cells which contain only a few or none at all, as is the case with many secretion cells (Figs. 13, 15).

The granules are perfectly spherical, appear quite homogeneous, and are highly refractive. They are hyaline, appearing under the microscope bluish or greenish by daylight. They greatly resemble droplets of fat. They are distributed throughout the cytoplasm of the cells and are frequently in active Brownian movement, though after standing some time they usually sink to the bottom of the cell, remaining there in a mass. The granules occur mostly as single spheres, though a great many of these are in pairs, clinging together in spite of Brownian movement or of violent currents in the preparation. Granules in such pairs appear slightly flattened at point of contact. This flattening is a refraction phenomenon, and is also seen when two granules accidentally come together, though it strongly suggests a division form, especially in view of the great number of such pairs which even on prolonged observation are almost never seen to become separated. In this connection it may be mentioned that there have been seen on several occasions forms as shown in Figure 27, the granules being elongate with a constriction at the middle. In such granules the material composing the two halves is seen to be continuous. While such forms are very rare in the honey-bee, in another of the Hymenoptera, *Halictus* sp., one specimen of which was examined, similar granules were found, and in nearly every epithelial cell, one to several such double granules were noted. The significance of these forms is not known. Hanging drops of epithelial cells and isolated granules in various solutions and culture media have been observed for periods of days, but no division or multiplication has ever been seen to take place. When the granules are allowed to stand in Locke's or other saline solution, a marked change in appearance usually occurs after a short time. The refractivity becomes somewhat lessened and the granules assume the appearance of hollow spheres containing a clear liquid or other substance (Fig. 29). This appearance is characteristic for the most part of the smaller granules. The outer covering of the larger ones frequently appears to be double (Figs. 28, 30).

The writer's experience confirms that of Koehler as concerns the failure of the granules to stain with most of the common histological stains. However, with Romanowsky stain, employing wet films fixed in osmic acid vapor and hardened in 70 per cent. alcohol, it is almost always possible to obtain preparations in which the granules appear embedded in the blue cytoplasm like perfectly clear, sharply defined vacuoles, containing each a deeply stained, blue-purple body, about half the diameter of the vacuole (Fig. 9). Similar results were obtained

with Giemsa. The staining even in the same mount, is frequently very irregular, for causes not understood. The deeply stained inner body is usually noted in those granules which are contained within an epithelial cell, while those isolated frequently lack the inner body. In general, the inner body retains the stain tenaciously throughout differentiation and dehydration in acetone or alcohol. Very rarely the entire granule stains solidly, or appears unstained in the center with a film of stain over the surface. The inner body is rarely at the center of the granule, but almost always lies touching one side. The problem of the nature of the granules remains unsolved. Nothing whatever is known of the method of their formation. Is the material deposited in this form by the cytoplasm, somewhat as crystals are formed, or does the organic portion of each granule, if such there be, bring about its formation after the fashion of plastids in plant cells? Are such organic portions self-reproducing or is each formed *de novo* by the cytoplasm? These and related questions can be answered only after much further study.

2. In addition to the larger granules just described, are frequently found very tiny refractive granules, spherical or somewhat irregular in shape, and measuring 0.2 to 0.5μ in diameter (Figs. 10, 12, 16-19, 24-26). These closely resemble the larger granules in appearance except for the irregularity in form. Even when irregular, they are approximately spherical. Rod-like forms are not uncommon and groups have been noted in which nearly all the granules were tiny rods, in length approximately one and one-half times the diameter. These small granules occur singly and in pairs. As is to be expected on account of their small size, they are in much more active Brownian movement than the larger granules, and are usually to be found actively dancing about long after the others have sunk to the bottom of the cell. The tiny granules have been considered as being distinct from the larger ones for several reasons, namely: their irregularity of form; the presence of rod-like forms; the fact that they are all of approximately the same diameter, tiny granules frequently being found in cells containing large granules with none intermediate in size; and further, the staining reaction to be mentioned presently.

Since the tiny granules were not considered separately until late in the present study, data obtained regarding their specific reactions are meager. In general their reactions are similar to those of the larger forms in that they are not to be found in sections and do not stain with common histological stains. No change is noticeable in the tiny granules after they have stood in salt solution. With Romanowsky and Giemsa following fixation with osmic acid vapor, they stain solidly and but for the absence of a surrounding vacuole, are indistinguishable from the deeply staining inner body of the larger

granules. In many cases, however, the granules fail to stain at all with this technique. The similarity in staining of the tiny granules and the inner bodies of the larger ones suggests the possibility that the former are the first stages in the formation of the larger granules. If such were the case, presumably these would become surrounded with calcium carbonate or other inorganic material and thus give rise directly to the larger spherical, refractive forms. Direct division of the tiny granules has not been observed, though the occurrence of elongate forms and pairs suggests that this may occur.

3. In a number of specimens taken from a hive in winter quarters, there were observed in addition to the refractive granules, irregular, slightly refractive bodies, measuring 1 to 1.5μ in diameter. While in such specimens these occurred in almost every epithelial cell, only rarely was there more than one in each cell. These irregular bodies could not be distinguished in stained cover-glass preparations or in sections. On account of the limited material further data were not obtained.

4. Toward the close of this study there was noted for the first time in bees taken during the spring from blossoms and from hives of different apiaries, the presence of slender rods in epithelial cells of the ventriculus (Figs. 21-23). These rods measured 0.1 to 0.2μ in diameter by 1 to 2μ in length. They were colorless and not refractive and could be distinguished from the cytaplasm in which they were suspended only with some difficulty. In this respect they resembled somewhat certain spirochaetes. The number to be found in any cell varied greatly. Many contained but very few, in which cases it was easy to observe the individual rods. These frequently exhibited slight Brownian movement. Other cells contained many rods which formed compact masses, usually at the periphery (Fig. 21). In such masses it was difficult to resolve the individuals. Many cells lacked these bodies, though they could be found in nearly all bees after prolonged search. In addition to the rods the refractive granules were present in apparently normal manner. Repeated efforts to stain the rods with Romanowsky, Giemsa and hematoxylin failed completely, not the slightest trace of them being found after staining, either in cover-glass preparations or in sections. The possibility that these bodies are needle-like crystals is not precluded.

5. Occasionally there are noted one to many spherical vacuoles in epithelial cells. They appear to consist of a liquid which is colorless or at times shows traces of pink. When present there are usually one to several vacuoles, about one-fifth the diameter of the cell. Rarely the cell is completely filled with small vacuoles. These are not to be confused with those surrounding the planonts of *Nosema apis* since no bodies can be made out within the vacuoles, either in fresh or

stained preparations. Since vacuoles have been observed more frequently and in greater numbers in bees from colonies suffering from various disorders, than in healthy bees, it is possible that the vacuoles indicate a pathological condition.

From the scope of the present study, the foregoing survey of the histology and cytology of the honey-bee's ventriculus has necessarily been in some respects a catalogue, rather than a discussion, of the many structures concerned. Their consideration has necessarily been almost entirely from the morphological point of view, and even in this respect much remains yet to be done, while the many related physiological problems, the solution of which is vital to an understanding of the whole subject, remain a practically unexplored field.

THE RELATION OF CYTOPLASMIC INCLUSIONS TO INTRACELLULAR MICRO-ORGANISMS

It is seen from the foregoing that in the cytoplasm of the epithelial cells are found a number of different bodies, of the nature, origin, behavior and ultimate fate of which it is not possible at this time to give a satisfactory explanation. A cursory examination of insect tissues in general reveals the fact that granules or other inclusions are characteristic not alone of the epithelial cells of the honey-bee's mid-intestine, but indeed of nearly every tissue of every insect. The same is true for many other arthropods. The further study of these inclusions becomes of immediate importance when the common occurrence in insects of intracellular parasites and symbionts is considered.

Intracellular parasitism in insects is of such common occurrence as to constitute an almost universal phenomenon. Many such parasites possess complicated life cycles and our knowledge of many is but fragmentary. Furthermore, in a number of cases in which organisms pathogenic for vertebrates pass a portion of their life cycle in the tissues of insects or arachnids, no trace of the organism has been found in the arthropod host. It is conceivable that the forms assumed by the organism in the arthropod tissues so closely resemble granules or other normal inclusions, and are thus so thoroughly masked, that they easily escape detection. That this may well be the case is borne out by the history of Rocky Mountain spotted fever, a tick-borne disease in which the causal agent is transmitted through many generations of ticks via the egg. The organism (one related to the Rickettsia group) has only recently been discovered by Wolbach (1919). He has described three forms of the organism in the tick, namely, tiny rods in the digestive tract, and two forms of short, paired rods or cocci in various tissues, one form being intranuclear. These organisms stain only with Giemsa. In another tick-borne disease, Texas cattle fever, in which the organism is transmitted via the egg to the second gen-

eration of ticks, the form present in the tick has not been described, although the form in the vertebrate host is well known. In certain tick-borne spirochaetoses there have been described or suspected granule stages of the organism in the tick (Leishman 1910, African relapsing fever; Balfour 1911, 1912, Hindle 1911, spirochaetoses of fowls. Marchoux and Couvy (1913) dissented from Hindle's view, claiming the granules found in ticks to be normal and not derived from spirochaetes.) The literature of Rickettsia in connection with typhus and trench fever, transmitted by lice, is suggestive. These organisms were held by several investigators to be merely normal granules (Wolbach, Todd and Palfrey 1922, bibliography). Tiny granule-like organisms of the Rickettsia type, both intra- and extra-cellular, some transmitted hereditarily, have been found in more than a dozen insects and ticks, and are apparently of general distribution throughout these groups. In his work on the etiology of yellow fever, Noguchi (1919) discovered that in one culture of the causal organism, *Leptospira icteroides,* the typical spiral form had disappeared, while in its place were many tiny granules. The spiral form was later recovered in transfers from this culture. Noguchi suggested that the organism may possess a granule stage in its life history—an especially interesting possibility in view of the fact that the disease is carried by an insect and the further fact that the form present in the insect has not yet been described. The few examples cited will serve to show that in many instances organisms in the tissues of insects can be recognized as such only with difficulty or not at all, and that as a result the many cell inclusions must be studied carefully and in some cases are rightfully to be placed under suspicion.

The problem is further complicated by the common occurrence of intracellular symbionts, certain organisms being constantly present in definite regions or organs or cells of various species of insects. These organisms are transmitted from generation to generation through the egg, and their development in the embryo and adult follows a course as definite as that of any organ of the insect itself (Buchner 1912, 1921, bibliography). Since in the species concerned there are no uninfected individuals for comparison, many symbionts now well known and easily recognized, were for some time believed to be merely cell inclusions. This view, for example, was held concerning the large, rod-shaped bacteroids of the Blattidae. The extent to which intracellular organisms have been overlooked and misinterpreted, and the probability that many symbionts or parasites of the granule-like Rickettsia type are yet to be described, make necessary great caution in passing judgment on the cytoplasmic inclusions of insect tissues.

Koehler (1920) considered the possibility of the refractive granules being symbionts, but abandoned the idea on finding them, as she

believed, to be composed almost entirely of calcium carbonate. There may be noted, however, a number of cases in which symbionts are found in the epithelium of the mid-intestine of other insects, notably the bacteroids of the carpenter ant, Camponotus, and yeast-like organisms in the "drug-store" beetle, *Sitodrepa panicea* (Buchner 1912), the pith-eating Lepidopterous larva, *Nonagria typhae* (Portier 1911), and certain of the pupiparous Diptera such as Glossina and Melophagus (Roubaud 1919). There has not been described any organism living in symbiosis with the honey-bee.

THE VENTRICULUS INFECTED WITH *NOSEMA APIS*

In this study of *Nosema apis* the chief purpose has been the determination of changes produced by the parasite in the host tissues, rather than a study of the parasite itself. A consideration of the latter, however, is a necessary prelude to a study of the pathological histology of the host. In the literature of the Microsporidian parasites found in honey-bees throughout the world, the organisms have in nearly all cases been identified as *Nosema apis* Zander. As noted below, there is strong probability that at least one other Microsporidian species may be present. Identification at best is somewhat uncertain because of the technical difficulties in determining accurately all the different stages in the life cycle, and further because of the conflicting accounts of various writers. The parasite most commonly found in the writer's material has been provisionally identified as *Nosema apis*.

The morphology and life-history of the parasite have been discussed in some detail by Zander (1911, 1921) and by Fantham and Porter (1912a, 1912b), while White (1919) has given much information concerning the resistance of the spores to chemical and physical agents. Kudo (1921) has added some notes concerning the morphology of the spore of *Nosema apis* and has recorded the occasional occurrence of an undetermined Microsporidian, possibly a new species of Nosema, in the ventriculus of the honey-bee.

The morphology and development of *Nosema apis* as described by Zander and Fantham and Porter, which is very similar to that given by Stempell (1909) for *Nosema bombycis* Nägeli, is reviewed below, together with observations of the writer.

The spore of *Nosema apis* is swallowed by the bee with food or water and enters the mid-intestine. Here the spore germinates, this process consisting of the discharge of the coiled polar filament through a pore at one end of the spore, followed by the issuance of the ameboid germ. According to Fantham and Porter this body contains two nuclei which appear as refractive spots. Each amebula gives rise to one or sometimes two uninucleate bodies, termed planonts, which move slowly about by means of pseudopodia. These may multiply in the

lumen of the intestine, giving rise to colonies of young planonts, "each of which moves about over the epithelial surface of the gut, and finally penetrates between the cells or directly enters them and becomes intracellular. . . . The method of penetration of a cell by a planont is most difficult of observation, though it has been seen in life on a few occasions." Unfortunately details of the actual process of penetration were not given. Neither Zander nor Fantham and Porter have taken into consideration the peritrophic membranes or the striated border in considering the movements of the planonts from the germinating spore to the penetration of the epithelial cells. Presumably the spores after they enter the intestine are contained within the innermost peritrophic membrane and are thus separated from the epithelium by several layers of these membranes. The latter are for the most part effective barriers to bacteria and food materials, and the method by which the planont penetrates them is not clear. Fantham and Porter stated that the planonts free in the lumen "stain fairly well with Romanowsky stains" but only moderately after they have become intracellular, and are distinguishable from the cell contents only with some difficulty. These investigators were able to distinguish planonts from other organisms such as yeasts "(1) by their movements, (2) the stainability of the nucleus, (3) by chemical tests, of which that for fungus cellulose is the chief. Planonts have no fungus cellulose." In addition to penetrating epithelial cells directly from the lumen, the planonts "may reach the haemocoel or body cavity of the bee and remain there in a resting condition for some time. They lose their motility temporarily, become rounded or oval and lie quiescent. After an interval their activity returns and from the haemocoel they retreat between the cells to the epithelium of the gut, which they gradually penetrate." These investigators found planonts and meronts in the blood of the bee, but their evidence for the return of the planont from the haemocoel to the epithelium, involving a second penetration of a well developed, chitinous basement membrane, was not indicated. White (1919) stated that "in infecting the stomach the parasite reaches the basement membrane but does not penetrate it."

Arrived within an epithelial cell "the active motile planont becomes passive, loses its pseudopodia and enters on a growing stage," which is followed by multiplication after a short time (Fantham and Porter). The parasite at this stage is known as a meront. The round or oval, uninucleate meront increases in size and divides most commonly by binary fission, though there may be produced chains of daughter meronts or large multinucleate meronts which later divide into typical meronts. In Zander's (1921) diagram and microphotographs, the meront stage is represented by "nests" of greatly elongate forms or

chains of as many as eleven individuals, the isolated oval forms being greatly in the minority. Elongate forms were not common and chains were not observed at all in the writer's material.

In distinguishing the planonts and meronts from normal cell inclusions the appearance of the parasites in the fresh condition is a particularly important consideration. Fantham and Porter stated that the planonts after they have become intracellular are very difficult to see either fresh or stained. Stempell (1909) found the same to be true of the planonts of *Nosema bombycis*. In the writer's experience the intracellular planont stage has never been made out with absolute certainty. Apparently this form is similar in density, refractivity, etc., to the protoplasm of the cell and stains with the same intensity. Fantham and Porter have not described the appearance of the living meront, though in their figures drawn from fresh preparations, meronts are shown as rounded or oval bodies, all with distinct nuclei and with finely granular, or in some cases alveolar, cytoplasm. In the writer's preparations the meronts were mostly spherical or oval, and greatly resembled vacuoles with a slightly refractive outer portion. The protoplasm of the meront was clear, homogeneous and apparently of about the same density as the cytoplasm of the host cell (Figs. 24-26, 33-38). Only very rarely could structures be seen within the meronts, these being interpreted as nuclei of the latter (Figs. 26, 32). Stempell (1909) reported that the meronts of both *Nosema bombycis* and *Thelohania mülleri* possess a pellicle-like outer layer of protoplasm. It is perhaps such a structure which appears as the slightly refractive covering of meronts of *Nosema apis*. The meronts are usually somewhat larger than the mature spores. Greatly enlarged multinucleate meronts as described and figured by Fantham and Porter have not been observed by the writer. Their Figure 47, evidently drawn from a fresh preparation, shows portions of four host cells containing five large multinucleate and two uninucleate meronts. In addition there are oval or elongate bodies of about the size of mature spores, which apparently represent the host nuclei. From the writer's observations the nuclei of the epithelial cells are almost always very much larger than Nosema spores, even the small nuclei of the regenerative cells being usually at least twice as large as the spores. The nuclei frequently appear as large spheres with a number of distinct spherical or somewhat irregular bodies suspended within (Fig. 3), closely resembling the large multinucleate meronts shown by Fantham and Porter. There might, as a result, be a possibility in the examination of fresh material, of mistaking a large meront for the host nucleus, and an abnormally small nucleus for a parasite, and vice versa.

There was occasionally encountered another form of meront which may not be that of *Nosema apis* but may be one stage of the undeter-

mined Microsporidian found by Kudo (1921). These forms were elongate bodies, some of which were constricted at the middle and nearly all of which were bent forming an obtuse angle (Figs. 39-48). The elongate, bent forms were usually highly refractive, resembling in this respect, the mature spore. No internal structure could be made out. The forms undergoing division were somewhat less refractive and usually contained at the constriction a distinct vacuole, which seemed also to be undergoing division (Fig. 48). Shorter forms with a vacuole at one end also occurred, together with short refractive forms without a vacuole, resembling the ordinary mature spore of *Nosema apis*. Whether these are spores or whether they are meronts which later become the refractive elongate and dividing forms with vacuoles, could not be determined from the limited material. Accompanying the forms just described were occasionally numbers of mature spores which were refractive and contained a large vacuole at one end, similar in this respect to the spores of Kudo's Microsporidian. That these forms, both meronts and spores, are distinct from *Nosema apis* is indicated by a characteristic staining reaction. With the Romanowsky stain used by the writer, the meronts and spores of *Nosema apis* stain blue with rarely any trace of red. On the other hand, the vacuolate forms contain a more or less irregular group of ruby-red granules within the vacuole. Infection by this form was almost always accompanied by infection with *Nosema apis,* small scattered areas being occupied by the vacuolate parasites. In one series of sections, for example, the epithelial cells were all heavily infected with *Nosema apis,* while one Malpighian tube contained exclusively forms with the brilliant red granules.

Following multiplication of the meronts within the cell, spore formation takes place. This process has been described in some detail by Fantham and Porter. Each ultimate meront undergoes a number of somewhat complex cytoplasmic and nuclear changes, resulting in the formation of a single spore with several nuclei and a polar capsule containing a long coiled thread, the polar filament, the whole being provided with a dense, refractive spore wall. Fantham and Porter stated that "when young the contents of the spores are finely granular and a single nucleus can be seen within them in life." Such forms have not been seen in the writer's preparations. The protoplasm of young spores is denser than that of meronts and appears homogeneous throughout except for the frequent occurrence of a vacuole at one or both ends as described by Fantham and Porter. At this stage there are discernible at times several cross striations in the protoplasm of both fresh and stained spores. According to both Zander and Fantham and Porter, these may represent the polar filament in process of formation. The vacuoles and cross striations are more marked after

the spores have remained some time in salt solution. In older and mature spores, the formation of the refractive spore wall makes examination of structural details impossible in the fresh condition. Fantham and Porter's detailed description of the structure of the mature spore differs in many important respects from that of Kudo (1921). The former represent the spore as containing a binucleate, girdle-like sporoplasm at the equator of the spore, surrounding the polar capsule with its coiled polar filament. There are in addition three accessory nuclei. Kudo's diagram shows a uninucleate, rounded sporoplasm at one end of the spore, the polar capsule being at the other end, with no accessory nuclei. It may be mentioned that with ordinary histological technique these details cannot be made out at all. The spores stain as shown in Figure 4, there being a clear space at one end, traversed by an axial thread, probably the base of the polar filament. The remainder of the spore contents, including probably the polar filament, forms a deeply staining, elongate triangular mass.

Spores contained in epithelial cells which are discharged into the lumen of the intestine, are eventually voided with the feces and may then serve to infect bees taking food or water thus contaminated. To what extent reinfection by parasites produced within the same ventriculus may take place, has not been determined. Fantham and Porter (1912b) believed this to be possible to a limited extent by means of mature spores. Zander (1921) believed that reinfection may take place by means of younger stages as well as spores discharged into the lumen. Maassen (1912) held that reinfection by means of mature spores was impossible, though it could probably be brought about by younger forms. Zander held that only by some method of reinfection could be explained the fact that frequently the entire epithelial layer is filled with parasites. This view is supported by a number of facts. It has been the writer's experience that in the summer and early fall the number of infected bees is small. About October or November it is common to find one infected bee in every twenty or thirty, and it is noteworthy that almost without exception such infected bees are very heavily infected, while no traces of parasites can be found in other bees from the same hives. Equal opportunity for ingestion of spores may be assumed for all bees of the same hive. The heavy infection of a few out of many uninfected individuals may be due to differential susceptibility, or to the spread of the parasites within the host following initial infection by relatively few spores. The latter would seem the more probable, and is borne out by the occurrence of areas parasitized exclusively by the undetermined Microsporidian, surrounded by areas containing only *Nosema apis*.

All accounts agree that infection with Nosema is most frequently found in workers, though drones and queens are susceptible. How-

ever, the drones and queens of even heavily infected colonies may escape infection altogether. As to the larvae, reports differ. White (1919) as a result of inoculation experiments concluded that the larvae are not susceptible. Fantham and Porter (1912b), however, reported finding meronts and occasionally spores in cells of the larval mid-intestine, and Maassen (1919) reported infection of the brood. The infection is usually limited to epithelial cells of the ventriculus, whether of workers, drones or queens, though in heavily infected specimens it is not uncommon to find the parasites within cells of the basal portion of the Malpighian tubes. Fantham and Porter (1912b) have reported the presence of planonts and meronts in the body fluid. Parasites have not been found in cells of the esophageal valve nor in those of the small intestine.

CHANGES IN THE VENTRICULUS PRODUCED BY NOSEMA

The presence of the parasites produces certain changes in the ventriculus which are recognizable with the naked eye. When the cells of the epthelium are filled with spores, the translucent, reddish brown appearance becomes a milky or chalky whiteness. On being crushed under a cover-glass the entire ventriculus may disintegrate forming a milky mass. White (1919) found that "the organ is often increased in size, the circular constrictions are less marked, and the transparency is diminished. In late stages of the disease, however, the stomach approaches the normal in size and the constrictions are again well marked." Swollen forms were not common in the writer's material. It is only in advanced stages of infection, however, that the presence of the parasites can be detected by the milky or chalky appearance, and not always with certainty even then. While normally reddish brown in color, the ventriculus of healthy bees varies from a whitish yellow to dark brown. When the epithelial cells of the lighter colored specimens are filled with large refractive granules, the outward appearance is somewhat similar to that of an infected ventriculus. Microscopic examination is the only certain method of determining the presence or absence of the parasites. The consistency of the ventriculus is markedly different in advanced stages of infection. Normally it is firm and when crushed under a cover-glass tends to recover its form. The peritrophic membranes remain together in a mass. The heavily infected ventriculus has lost its firmness and elasticity and disintegrates readily. Many cells filled with spores remain intact, but an enormous number of spores escape from the easily ruptured cells. Peritrophic membranes are rarely recognizable as such.

Changes in the general shape and arrangement of epithelial cells and other structures due to the presence of Nosema become apparent

only after the organisms have been fairly well distributed throughout the intestine and the majority of cells have become infected. Newly infected cells, except for certain cytoplasmic features, appear to be quite similar to their uninfected neighbors (Fig. 3). Infection may proceed to a stage where the majority of the cells contain a number of meronts, or even spores, before any differences are noted other than the presence of the parasites themselves. This is true of fresh as well as stained tissues. The size and shape of the cell and the appearance of the cytoplasm are unchanged. The striated border and peritrophic membranes are normal. The formation and discharge of secretion cells proceeds as usual. The small, stalked secretion cells which have been budded off from the larger epithelial cells may or may not contain meronts or spores.

After the epitelium has been completely parasitized and the infection has persisted some little time, a number of marked changes are recognizable. The musculature and basement membrane alone are unaffected. The epithelial cells are in general larger, evidenced by their greater length in sections, and increased diameter as seen in fresh preparations. Fantham and Porter (1912b) stated that "the passage of the spores outwards into the lumen of the gut causes tears and gaps to appear in the intestinal wall," such injured cells being replaced by new ones. Liberation of spores in this fashion has not been observed by the writer, the parasites practically always being within host cells until the latter have left the epithelium and have disintegrated in the lumen. These writers further stated that "when an intense infection is present, the bee seems to lose its power of reforming cells." Quite the reverse would seem to be true from the writer's experience. There is a marked tendency toward increased proliferation of cells in heavily infected individuals, resulting in an abnormally thickened epithelium, composed of greatly elongate cells attached to the basement membrane and occasionally there are elongate secretion cells arising from these (Fig. 7). This condition may also be explained by delay in the discharge of secretion cells into the lumen. Excessive proliferation and elongation of cells are by no means invariable accompaniments of heavy infection, nor are they found only in infected tissues. In many infected areas the cells are not at all elongate. Figures 6 and 7 represent different regions of the same section, the cells of one being enormously elongate, while those of the other are quite the reverse, the degree of infection being the same in both. One method of formation of secretion cells is strikingly shown in Figure 7. Small cap-like cells are seen covering the ends of several epithelial cells, the former being entirely free of parasites, while the parent cells are filled with them. This method of secretion-cell

formation, as already described, has also been observed in uninfected specimens, which indicates that even heavily infected cells may function normally in some respects.

In heavily infected areas Fantham and Porter (1912b) found "the secretory epithelium reduced to the condition of a pulp or sponge-like meshwork, enclosing large colonies of meronts and spores within its strands." Degeneration of the cells to this extent has not been noted in the writer's material. With the exception of the region next the basement membrane, the cell outlines are usually clear (Figs. 4-7). The nidi, the cells of which may contain few or no parasites, even in heavily infected individuals, may be little changed or, on the other hand, may not be recognizable at all, as shown in Figures 6 and 7, drawn from different areas of the same section.

With advance in the degree of infection, the striated border becomes less marked. In fresh mounts the epithelial cells filled with spores usually lack the radiating hair-like processes so conspicuous in the case of healthy bees. In sections the hair-like structure may be quite definite in some areas, in others there may be merely a granular mass (Figs. 5-7). Along with the striated border, the peritrophic membranes become more indefinite and their formation more uncertain as infection progresses. Frequently the peritrophic membranes are scarcely recognizable, the lumen of the ventriculus being entirely filled with spores, either free or within discharged cells. In such sections the space between the spores may be occupied by a finely granular substance, together with bacteria and yeasts. Maassen (1919) noted the occurrence of greater numbers of bacteria in infected than in uninfected individuals.

The morphologically recognizable effects of Nosema upon the contents of the epithelial cells are few. The host nuclei appear not to be affected, since in heavily infected cells they are not appreciably different from those of normal cells. Parasites within host nuclei have not been mentioned by the various investigators, nor has this condition been noted by the writer. This point, however, is difficult of determination with certainty, for in many heavily infected cells there are no nuclei. It may be that in such cases the nuclei have been destroyed, or on the other hand, the cells may be secretion cells which never possessed nuclei.

Fantham and Porter (1912b) found a clear space or "halo" surrounding meronts a short time after they had become intracellular, this halo becoming more marked with the growth of the meront. These writers considered the clear space to be possibly an alteration of the concentration of the liquid surrounding the parasite, due perhaps to the protoplasm having been digested by the parasite, or "to the removal by simple absorption from the cytoplasm of the invaded

cell of various granular constituents, used by the parasite as food." It is not clear whether the "granular constituents" refer to the conspicuous cell inclusions, or to the protoplasm itself which is at times indistinctly finely granular. Clear spaces or vacuoles surrounding the meronts have been observed but rarely by the writer in fresh material, though in sections a rather definite space may be found surrounding each parasite (Fig. 4). Both Zander (1921) and Fantham and Porter (1912b) have figured "nests" of meronts lying within large clear spaces.

The effect of *Nosema apis* on the various cell inclusions has received little attention. Fantham and Porter have not mentioned these bodies specifically, nor do the latter appear in their figures drawn from fresh preparations, though large numbers of the refractive, spherical granules appear in certain of their microphotographs (1912a). Zander (1921) gave a figure of a healthy cell filled with "Kalkkörperchen" or refractive granules, and showed a few of these in a cell filled with spores. Koehler (1920), who was led to the investigation of the epithelial inclusions in connection with a study of Nosema-infected bees, stated that in cells filled with spores the "calcium-granules" are few or have disappeared altogether. She suggested that the pathological effect of the parasite, while it might be the effect of toxins, might also be due to a disturbance of digestion and resorption resulting from a deficiency in calcium. On testing for calcium in the spore walls, Koehler obtained only negative results, so that apparently the calcium of the granules is not used directly by the parasites. However, she considered the disturbance of the calcium cycle a probability.

The writer's observations agree with those of Koehler as to the decrease in number of the refractive granules in heavily infected cells. It is only rarely, however, that the larger, nucleate cells lack these entirely, though the smaller secretion cells are often without granules. There is no change in appearance or staining reaction of these granules, though a few abnormal forms have been noted (Fig. 31). There is, however, in nearly all cases a very marked increase in the number of the very tiny granules described above (p. 123). These are spherical, slightly irregular or rod-shaped, and very frequently in pairs. In infected specimens it often happens that such tiny granules predominate even in cells which contain no parasites. With Romanowsky these tiny bodies may stain deeply, though quite as frequently they are not to be found after staining, or else their numbers are greatly reduced. Whether these tiny granules represent the central portion of the larger, refractive granules minus their inorganic outer portion, and are thus a degeneration stage of the latter, cannot be stated. It would seem certain, however, that their marked increase

in number is due in some way to the presence of the parasites. The appearance of the cytoplasm is unchanged, though it is, of course, ultimately replaced in large measure by the parasites. The slender, non-refractive rods (p. 124) have been observed in the epithelial cells of a few heavily infected individuals.

SUMMARY OF PATHOLOGICAL CONDITIONS ASSOCIATED WITH NOSEMA

From the foregoing it is seen that throughout infection the epithelial cells retain their identity, the cells not being "destroyed" at all, strictly speaking, since they disintegrate only after they have reached the lumen. Though the cytoplasm is largely replaced by parasites, the nuclei and cell membranes seem uninjured, though the latter are probably more easily ruptured. Along with destruction of the cytoplasm, changes in relative numbers of various cell inclusions take place. There appears a tendency toward increased proliferation of epithelial cells, with consequent thickening of the epithelium. With the advance in infection, the formation of striated border and peritrophic membrane becomes seriously deranged. Changes in the appearance and contents of the organ harboring the parasites occur only with heavy infection. The color changes from red or brown to chalky white, and the firmness and elasticity of the tissues are lost.

From the behavior of infected bees it is seen that these pathological conditions do not immediately produce outward symptoms of disease. In a colony known to harbor the parasites, it is impossible to distinguish from appearance or behavior, the infected from the uninfected individuals, except those actually dying of the disease. Since in this latter condition the usual symptoms, i. e., crawling, inability to fly, distended abdomen, etc., are quite as characteristic of disorders not associated with Nosema, microscopic examination is the only certain method of diagnosis. Since it is thus possible for the infection to be in an advanced stage without having any apparent effect on the behavior of the bee, the ultimate pathological effect, namely, the weakening and death of the bee, would appear to be due, not to any one of the pathological conditions enumerated above, but to the collective and cumulative effect of some or all of them. Toxins produced by the parasite, if any, would seem to make little or no contribution to the pathological condition, since their effects could be expected to manifest themselves during the growing stages of the parasite. Until more is known of the physiology of the honey-bee and of insects in general, the most plausible explanation of the condition, and the one commonly advanced, is that some derangement of the digestive processes takes place, which leads to the malnutrition and hence the weakening and ultimate death of the host.

A PATHOLOGICAL CONDITION OF THE VENTRICULUS NOT ASSOCIATED WITH NOSEMA

In June 1920 there came to the notice of the writer a hive which had been suffering a marked and constant loss of bees since it was set out in the spring. There were generally to be found on the ground near the hive as many as a hundred or more bees which were in great distress, crawling about excitedly or sluggishly, or lying motionless except for occasional trembling movements of wings and legs. Examination failed to reveal the presence of Nosema and there was no apparent cause for the death of the bees in such numbers. The colony was an isolated one, located in the residential district of St. Anthony Park, St. Paul, about one-quarter of a mile from the apiary at University Farm. No marked loss of bees was noted in the latter apiary.

The mid-intestines of the diseased bees examined in June 1920 were mostly pale yellowish, and decidedly smaller in size than normal. The contents were colorless or pale brown. The hind-intestine had a pale, watery appearance. When the ventriculus was crushed under a cover-glass, the epithelial cells which became detached lacked the elasticity of normal cells. They tended to retain the elongate-pyriform shape instead of becoming spherical when the pressure of adjoining cells was released. Apparently normal granules were present in the cells, though in some specimens there were in addition refractive spheres or globules, possibly of some liquid, very much larger than the ordinary refractive granules. Sections revealed a rather striking pathological condition, totally unlike that encountered in Nosema-infected bees. The entire cellular structure had in many regions become a coarsely granular mass. The outlines of many cells and nuclei were wholly indefinite (Fig. 8). In certain regions there appeared to have taken place an excessive and irregular proliferation of cells, the mass of discharged cells forming a layer thicker than the epithelium itself. Parts of the epithelium seemed to have been shed into the lumen *en masse,* the more or less intact epithelial layer being separated from the basement membrane by a considerable area filled with coarse, deeply staining granules. In certain cases, as shown in Figure 8, the epithelium had been given off in a body, but a new epithelium had been formed beneath the old. As a result there were two layers of epithelium, both showing the degenerate, granular condition. The inner layer was broken or had completely degenerated in several places, but portions of the striated border were present together with a peritrophic membrane in process of formation, and several of the latter in the lumen completely formed. On the surface of the layer resting on the basement membrane, was an unbroken striated border with the beginning of a peritrophic membrane. The basal por-

tion of this epithelial layer had lost all trace of cell outlines, being a uniformly granular area. The Malpighian tubes and muscle fibers surrounding the ventriculus were also degenerate.

Further examination of bees from this colony was not made in the season of 1920, though the owner stated that the loss of bees continued to a certain extent throughout the entire summer. The colony yielded little or no surplus honey, but was able to winter over. Shortly after it was set out in the spring of 1921 a similar loss of bees was noted. The number of stricken bees to be found at any one time, however, was rarely over forty or fifty and at times not more than a dozen. On examination the ventriculus was found to be pale and translucent, with a dark mass at the posterior end formed by the contents. The ventriculus was usually of less than normal size and was frequently of uneven diameter. Within the epithelial cells were found large numbers of the non-refractive, slender rods described above (p. 124). Since these rods were also found in healthy bees from other apiaries, no pathological significance was attached to them. In addition there were noted the abnormally large refractive bodies found the previous summer, and also a number of vacuoles. One apparently healthy bee, taken from the entrance, was heavily infected with *Nosema apis*. This parasite was not found in any other individuals, whether active or crawling. In the few specimens sectioned, the striking pathological condition found the previous season was not apparent. The only abnormal structures noted were occasional large spheres, staining lightly but uniformly with eosin. The loss of bees continued until shortly after July 1, 1921, about which time the colony was requeened.

The cause of this pathological condition, and whether or not it is infectious, are not known. It is merely one more example of the many disorders of the adult honey-bee which cannot be distinguished from each other with certainty until our knowledge of insect physiology, and of the normal and pathological histology, not alone of the mid-intestine, but of all other organs as well, is greatly extended.

The writer gratefully acknowledges the aid and encouragement of Professor C. W. Howard, under whom the work was begun, and of Dr. William A. Riley, under whose direction it was continued.

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Explanation of Figures

Figures 1 to 9 drawn with aid of camera lucida from stained preparations; Figures 10 and 49 drawn with aid of camera lucida from fresh material.

Explanation of Plate IX

1. Normal ventriculus, longi-section. Gilson. x 350.

2. Dividing nucleus of regeneration cell. Gilson. x 790.

3. Cross-section of epithelium, one cell containing meronts of *Nosema apis*. Vacuoles in cytoplasm represent refractive granules of fresh tissue. Osmic acid vapor. x 790.

4. Cells containing meronts and spores surrounded by clear areas in cytoplasm. Gilson. x 770.

5. Longi-section, junction of heavily infected ventriculus with small intestine; base of Malpighian tube slightly infected. Infected area ends abruptly with beginning of small intestine. Gilson. x 180.

6. Cross-section, heavily infected ventriculus; shape and arrangement of cells nearly normal. (Compare with Figure 7). Sublimate-alcohol. x 180.

7. Cross-section, same specimen as Figure 6. Epithelium enormously thickened as result of elongation and increase in number of cells; several cap-like secretion cells, some with cross-walls; nidi not recognizable as such. x 180.

8. Cross-section, ventriculus; pathological condition not associated with *Nosema*. Two epithelial layers apparently due to sloughing and regeneration: sloughed portion, *A*, nearly intact with striated border and peritrophic membrane; newer epithelial layer, *B*, resting on basement membrane, degenerate but with striated border and peritrophic membrane forming. Malpighian tube, *m. t.*, and muscle fibers, *m. f.*, degenerate. Hollande. x 325.

9. Refractive granules from epithelial cell, cover-glass preparation, osmic acid vapor, Romanowsky stain. Granules appear as vacuoles embedded in blue cytoplasm, each with blue-purple inner body. x 1120.

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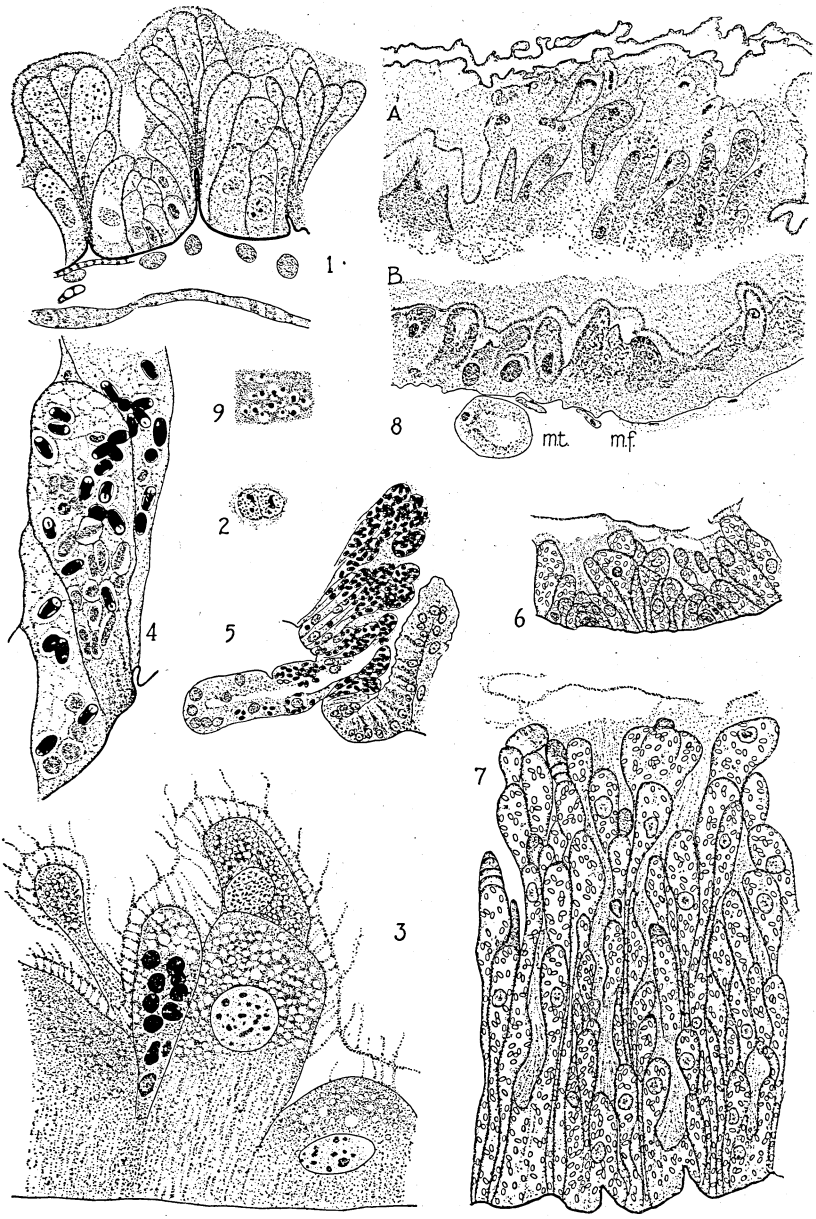


PLATE IX

EXPLANATION OF PLATE X

10. Isolated epithelial cell with striated border. The cytoplasm contains large, spherical, refractive granules and tiny, somewhat irregular granules. x. 1910.

11-18. Secretion cells. x 1910.

19, 20. Stalked secretion cells with striated border. x 1910.

21-23 Epithelial cells containing slender, non-refractive rods in addition to normal, refractive granules. Figures 21 and 22, x 1910; Figure 23, x 1810.

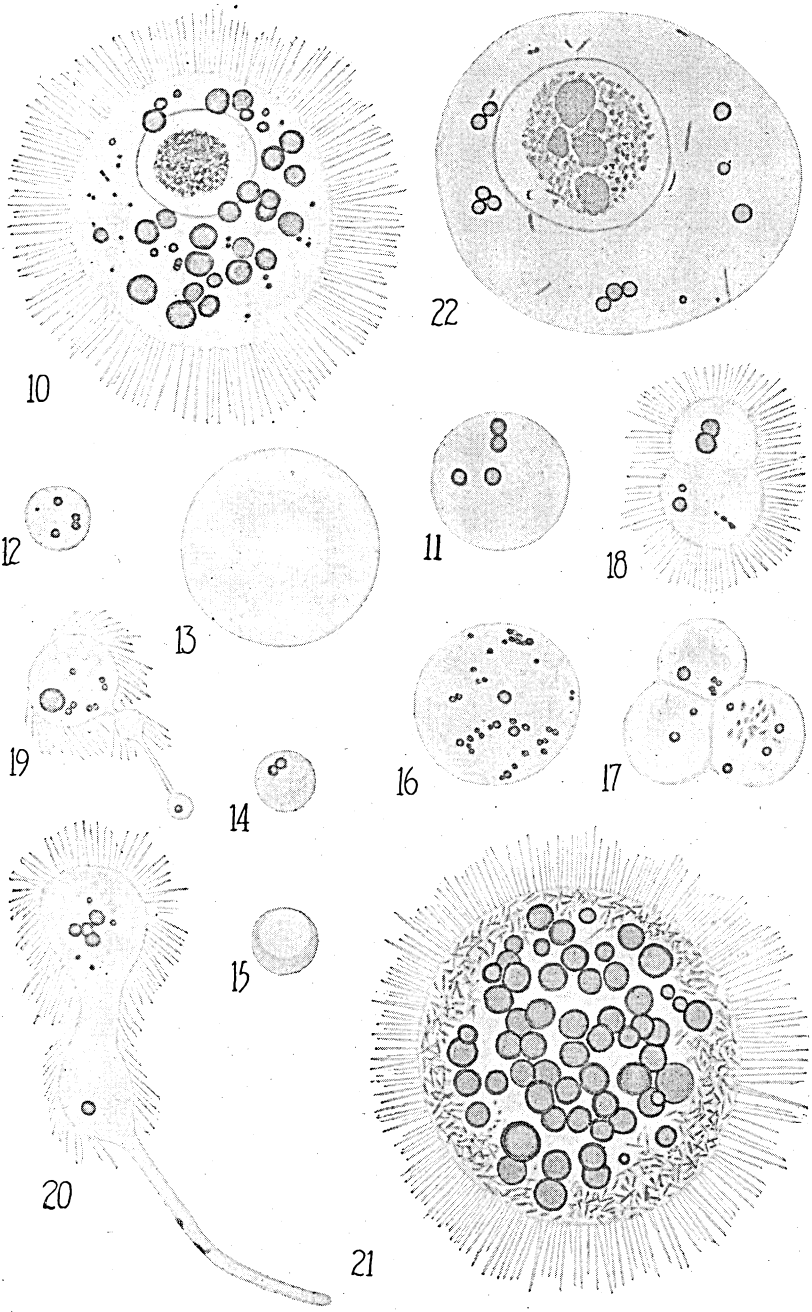


PLATE X

Explanation of Plate XI

24, 25. Epithelial cells containing meronts of *Nosema apis* and many tiny, irregular granules. x 1810.

26. Epithelial cells containing spores and meronts, the latter resembling vacuoles. The cytoplasm contains spherical, refractive granules and tiny, irregular granules. x 1810.

27. Double, refractive granule from epithelial cell, suggesting a division form of the spherical, refractive granules. x 1810.

28-30. Refractive granules after standing in Locke's solution one to two hours. x 1810.

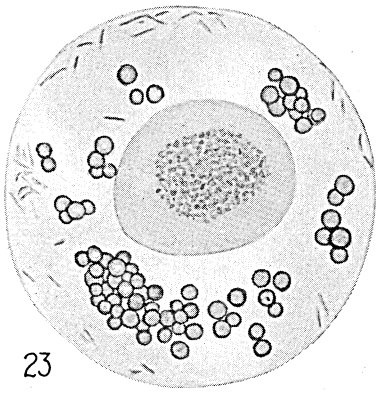
31. Abnormal forms of refractive granules from heavily infected epithelial cells. x 1810.

32-38. Young meronts of either *Nosema apis* or undetermined Microsporidian. x 1810.

39-48. Meronts, probably of undetermined Microsporidian. x 1810.

49. Young spore, *Nosema apis*. x 1810.

50. Epithelial cell filled with spores, together with a number of spherical, refractive granules. Microphotograph, fresh preparation. x 890.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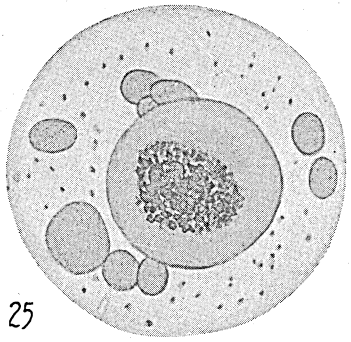
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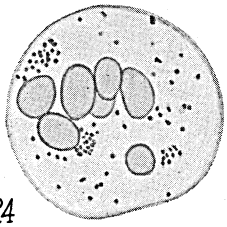
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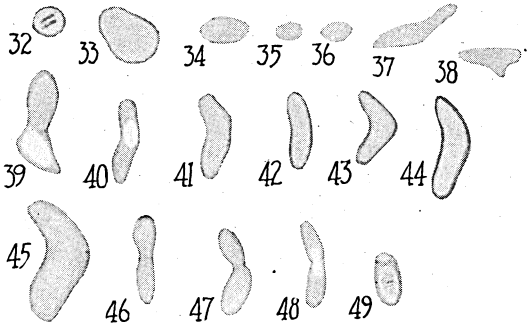
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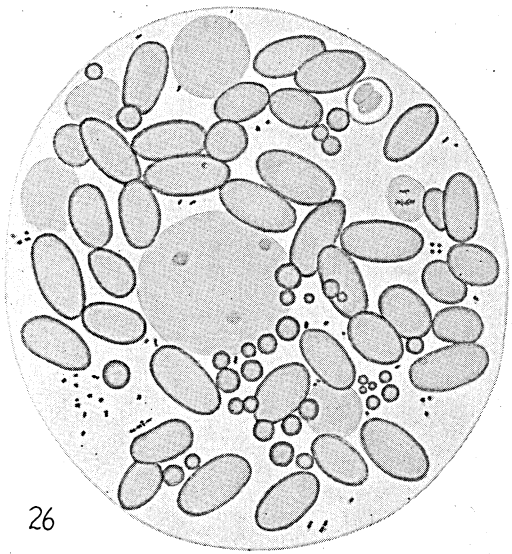
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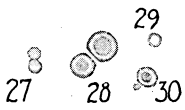
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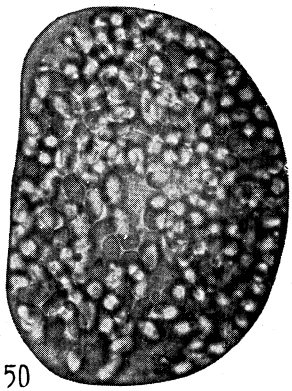
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PLATE XI

TECHNICAL BULLETIN 14 March 1923

The University of Minnesota
Agricultural Experiment Station

The Parasitism of Colletotrichum lindemuthianum

By J. G. Leach
Division of Plant Pathology and Botany

UNIVERSITY FARM, ST. PAUL